

A comparative study of ejaculate traits in three endangered ungulates with different levels of inbreeding: fluctuating asymmetry as an indicator of reproductive and genetic stress

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We studied three closely related species of endangered gazelles (*Gazella dorcas*, *Gazella dama* and *Gazella cuvieri*) with different levels of inbreeding in order to determine at which intensities inbreeding influences ejaculate traits. We also examined whether fluctuating asymmetry (FA) is a reliable indicator of genetic as well as reproductive stress. Our results show that, within each population, the individual coefficient of inbreeding is inversely related to ejaculate quality only in the species with the highest levels of inbreeding (*G. cuvieri*). In addition, FA is a reliable indicator of individual levels of inbreeding in both the species with the highest levels of inbreeding (*G. cuvieri*) and the species with intermediate levels of inbreeding (*G. dama*). Thus, FA appears in individuals whose levels of inbreeding are still not high enough to affect male reproductive potential and should therefore be considered a sensitive indicator of genetic stress. Finally, FA is also a reliable indicator of male reproductive stress since it is related to individual semen quality in all the species studied.

Keywords: inbreeding; fluctuating asymmetry; ejaculate; spermatozoa; ungulate; gazelles

1. INTRODUCTION

Given the widespread decline and fragmentation of animal populations, there is an urgent need for fully understanding the negative consequences of small population size and lack of genetic flow. Recovery plans for endangered species sometimes include breeding programmes which must begin with a small number of founders, either because there are no more individuals left in the wild or because it is not advisable to extract too many individuals from the remaining natural populations. Under these circumstances it is also crucial to have a better understanding of the genetic problems arising in small populations in order to implement management plans which will render the best possible results.

Many studies on plants and laboratory and domestic animals, as well as zoo populations, have shown that inbreeding reduces fitness (Charlesworth & Charlesworth 1987; Thornhill 1993). However, it has been much more difficult to establish whether inbreeding has any impact upon natural populations given the difficulties in reconstructing pedigrees over several generations. Such lack of information has led some authors to question the importance of genetic factors in relation to demographic and environmental stochasticity (Shields 1993; Caro & Laurenson 1994; Caughley 1994), giving rise to heated debate (Lande 1988, 1993; Harcourt 1991; Mills & Smouse 1994; Frankham 1995a,b; Parsons 1996).

More recently, the results of longitudinal studies of vertebrate populations have generated contradictory results (Van Noordwijk & Scharloo 1981; Gibbs & Grant 1989; Hoogland 1992; Grant & Grant 1995; Keller 1998). Two different factors may help explain these discrepancies. First, studies which have covered periods in which

the environmental conditions were particularly harsh have shown that during these periods the effects of inbreeding become most apparent (Keller *et al.* 1994). Thus, genetic and environmental conditions interact and the negative consequences of inbreeding may remain hidden until the environmental conditions deteriorate. Second, these studies have assigned paternity on the basis of observed copulations, but this approach is flawed in those cases in which females mate with more than one male.

The use of molecular techniques in assigning paternity has overcome this latter problem. Recent studies which have measured the genetic similarity between parents in natural populations have shown that inbreeding has a negative impact upon offspring survival in birds (Bensch *et al.* 1994; Kempenaers *et al.* 1996), as well as mammals (Stockley *et al.* 1993). These studies have shown that mating between close relatives takes place even in natural populations with low levels of inbreeding and that, when it occurs, it does reduce fitness.

The genetic basis underlying inbreeding depression is related to a decrease in heterozygosity (Charlesworth & Charlesworth 1987; Thornhill 1993; Dudash & Carr 1998). Thus, a more direct approach has been to measure levels of heterozygosity. This was initially done by using alloenzymes but, although these studies found positive relationships between the levels of heterozygosity and fitness, the methodology has been criticized because of the low number of loci used and for the low number of segregating alleles (reviewed in Allendorf & Leary 1986; Mitton 1993). The use of microsatellites has proved to be a more valuable tool in this context. A recent study on a natural population of harbour seals (*Phoca vitulina*) showed that individual heterozygosity is related to birth weight and to survival before weaning (Coltman *et al.* 1998).

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While the evidence supporting the idea that inbreeding reduces fitness seems compelling, it is not enough to resolve the most controversial topic, i.e. whether inbreeding can lead to the extinction of populations. A very recent study carried out in a butterfly metapopulation consisting of numerous small local populations showed that the extinction risk increased significantly with decreasing heterozygosity after accounting for the effects of ecological factors (Saccheri *et al.* 1998). Such drastic effects can be rapidly reversed by introducing new individuals, as shown in a population of adders which suffered from severe inbreeding depression (Madsen *et al.* 1999).

Given the important contribution of genetic factors to the decline of populations and, eventually, to their extinction, it is crucial to determine which fitness components underlie this relationship. Most studies have found a decrease in offspring survivorship either during embryo development or shortly after birth (Ralls *et al.* 1979; Quattro & Vrijenhoek 1989; Bensch *et al.* 1994; Kempenaers *et al.* 1996; Lacy *et al.* 1996; Margulis & Altman 1997; Coltman *et al.* 1998; Coulson *et al.* 1998; Saccheri *et al.* 1998; Eldridge *et al.* 1999). While effects on female fecundity or offspring mortality are easy to detect, our knowledge of the influence of inbreeding upon male fitness is still poor. The widespread occurrence of female promiscuity and, thus, sperm competition (Birkhead & Møller 1998) has been a recurrent problem in studies which have tried to determine the effects of inbreeding on male reproductive success. A more fruitful approach has been to study the effects of inbreeding upon male reproductive physiology. Comparative studies between carnivore populations with differing levels of genetic variability have suggested that inbreeding decreases the male reproductive potential (O'Brien *et al.* 1983, 1985, 1987; Wildt *et al.* 1983, 1987; Menotti-Raymond & O'Brien 1993). These studies gave rise to an intense debate because critics argued that the conclusions were flawed due to the lack of a control population with high genetic variability, the use of alloenzymes and the fact that interpopulation comparisons could not control for confounding effects. A recent study on an endangered ungulate with high levels of inbreeding has shown that, within a population, inbreeding at the individual level is negatively related to semen quality (Roldan *et al.* 1998). Given that, in this population, the environmental factors were common to all individuals, the relationship between the level of inbreeding and ejaculate quality is likely to be causal.

It has been argued that inbreeding could also decrease developmental stability (Lerner 1954), but the evidence seems contradictory. More specifically, it has been suggested that the level of heterozygosity should be related to the levels of fluctuating asymmetry (FA), which refers to small random deviations from perfect bilateral symmetry in morphological characters and reflects the ability of the individual to withstand stress during the development of a trait (Van Valen 1962; Palmer & Strobeck 1986; Parsons 1990, 1992; Møller & Swaddle 1997). Because selection against asymmetry will be strong upon characters which are essential to the normal functioning of an individual, it is likely that FA will be particularly marked in sexual ornaments which show high variability

between individuals and are costly to produce (Møller & Swaddle 1997; Møller & Thornhill 1998). The evidence so far seems contradictory (Sheridan & Pomiankowski 1997; reviews in Markow 1995; Møller & Swaddle 1997; Clarke 1998; Møller 1999).

Part of the reason why the evidence available seems difficult to integrate is that the results come from populations which differ in their average levels of inbreeding and in the ranges covered, making comparisons between studies difficult. It seems obvious that the effects of inbreeding will become more serious as heterozygosity decreases. Perhaps more importantly, causal effects can only be established when there is enough variation in the levels of inbreeding within populations. In order to understand these discrepancies we need to compare closely related species that are subject to different levels of inbreeding and that have enough intraspecific variation.

The aim of our study was to investigate whether inbreeding affects semen quality and horn FA in three closely related species of gazelles which differ both in their average levels of inbreeding and in the ranges of coefficients of inbreeding present in each population.

2. MATERIAL AND METHODS

(a) *Study population*

Since 1971, the Estación Experimental de Zonas Áridas (CSIC) has developed a successful captive breeding programme for three endangered gazelle species: *Gazella dama mhorr* Bennet 1833, *Gazella dorcas neglecta* Lavauden 1926 and *Gazella cuvieri* Ogilby 1841. For the history of the populations see Alados *et al.* (1988). The sizes of the founding populations differed between the three species (*G. dorcas* 11:13, *G. dama* 3:9 and *G. cuvieri* 2:2). In the present study, a total of 19 males of *G. dorcas*, 17 males of *G. dama* and 14 males of *G. cuvieri* were used, all of them healthy and reproductively mature.

(b) *Coefficient of inbreeding*

Paternity could be reliably assigned for all offspring because only one breeding male is present with each group of females. This allowed the reconstruction of whole pedigrees and the calculation of individual coefficients of inbreeding following the additive relationship method (see Wright 1922; Ballou 1983). Despite efforts aimed at minimizing the number of matings between related individuals, it has been difficult to avoid inbreeding due to the small number of founders in each population (see §2(a)). The individual coefficients of inbreeding varied within each species, thus allowing intrapopulation comparisons. The individuals included in the sample covered the whole range of coefficients of inbreeding for each species.

(c) *Semen collection and analysis*

Semen was collected from male gazelles of the three species by electroejaculation under surgical anaesthesia (see Cassinello *et al.* (1998) for details) in October–December 1996 and October–December 1997. This time of the year was chosen because there is a peak in mating activity in *G. cuvieri* (Olmedo *et al.* 1985); the other two species do not follow a seasonal pattern.

The methods used to assess the ejaculate parameters have been described previously (Cassinello *et al.* 1998). Briefly, the sperm concentration, motility and progressive motility were assessed within 60 min of collection. Aliquots were diluted in a

modified Tyrode's medium and used to assess motility and subsamples taken to analyse the sperm morphology and acrosome integrity. Motility was evaluated subjectively. The sperm morphology (structural abnormalities) and acrosome integrity were quantified by staining sperm with eosin-nigrosin and then with Giemsa (Tamuli & Watson 1994). The sperm morphology was categorized as either normal or with abnormalities in the various sperm components. For acrosomal integrity, the percentage of spermatozoa with a normal apical ridge (NAR) was assessed. For each preparation 100–200 spermatozoa were counted. Thus, five ejaculate parameters were used in this study: the sperm concentration, percentage motility, percentage of spermatozoa with progressive motility, percentage of normal spermatozoa and percentage of spermatozoa with NARs.

(d) Phenotypic analyses

Body weight was obtained during anaesthesia just before electroejaculation. For the analysis of horn asymmetry and tests of FA, horn measures were always taken by the same operator (J.C.). Three measures were taken on the left and right horns of the males included in the study sample: (i) length (to the nearest millimetre along the horn on the front side using a flexible ruler), (ii) greatest latero-medial diameter at the base, and (iii) greatest oro-aboral diameter at the base (the latter two to the nearest 0.1 mm with a caliper). One male of *G. dama* and another one of *G. cuvieri* were excluded from the analyses of FA due to marked horn deflections. To test for FA, horn measures were also taken from the skulls of 45 sexually mature males from the three species. The repeatability of all the measures was very high, which indicated that measurement errors were insignificant for both the size and asymmetry measurements. No directional asymmetry or anti-symmetry were detected; the characters examined exhibited FA (see Roldan *et al.* 1998).

(e) Statistics

When parametric tests were used, the usual transformations were applied on non-normal variables (see Zar 1984). Relationships between the coefficient of inbreeding and ejaculate traits were analysed by simple regression analysis on transformed variables, as were the relationships between the coefficient of inbreeding and degree of FA. Spearman's rank correlations, corrected for ties if applicable, were used to analyse the relationship between the degree of FA and ejaculate quality.

3. RESULTS

(a) Coefficients of inbreeding: inter- and intra-specific variation

The different numbers of founder individuals of each of the three species (see §2(a)) led to differences in the average coefficients of inbreeding of the gazelle species breeding at the EEZA (table 1). *G. dorcas* showed the lowest mean values of inbreeding of the three species, *G. dama* exhibited intermediate values and males of *G. cuvieri* experienced the highest level of inbreeding of the three species ($F_{2,47} = 27.74$ and $p < 0.0001$) (Fisher's protected least-significant difference post-hoc test: *G. dorcas*–*G. dama* $p < 0.0001$, *G. dorcas*–*G. cuvieri* $p < 0.0001$ and *G. dama*–*G. cuvieri* $p = 0.02$). Within each species, the ranges of inbreeding coefficients were also different, with

Table 1. Average and ranges of inbreeding coefficients of individuals used in this study (*Gazella spp.*)

	sample size	inbreeding	
		mean \pm s.e.	range
<i>G. dorcas</i>	19	0.0228 \pm 0.0061	0.0000–0.0781
<i>G. dama</i>	17	0.1039 \pm 0.0164	0.0000–0.3137
<i>G. cuvieri</i>	14	0.1473 \pm 0.0144	0.0620–0.2260

G. dorcas having a limited range and *G. dama* and *G. cuvieri* having a wider range of inbreeding coefficients (table 1).

(b) Relationship between inbreeding and ejaculate traits

The intraspecific analyses showed no relationship between the coefficient of inbreeding of individuals and each of the five ejaculate traits in *G. dorcas* or *G. dama*. On the other hand, males of *G. cuvieri* with high levels of inbreeding exhibited a low reproductive potential: the coefficient of inbreeding was negatively related to four of the ejaculate parameters: the percentage of motile sperm ($n = 14$, $r^2 = 0.31$ and $p = 0.04$) (figure 1a), percentage of spermatozoa with progressive motility ($n = 14$, $r^2 = 0.46$ and $p = 0.01$) (figure 1b), percentage of spermatozoa with intact acrosomes, percentage of NARs ($n = 14$, $r^2 = 0.30$ and $p = 0.04$) (figure 1c) and percentage of normal spermatozoa ($n = 14$, $r^2 = 0.48$ and $p = 0.01$) (figure 1d).

(c) Relationship between fluctuating asymmetry and inbreeding

The analysis of inbreeding in relation to horn FA revealed a positive relationship between the coefficient of inbreeding and FA at the greatest oro-aboral diameter at the base of the horn in two species, *G. dama* ($n = 16$, $r^2 = 0.25$ and $p = 0.049$) and *G. cuvieri* ($n = 13$, $r^2 = 0.38$ and $p = 0.02$), but not in *G. dorcas*, the one with the lowest coefficients of inbreeding.

(d) Relationship between fluctuating asymmetry and ejaculate traits

The degree of FA was correlated with ejaculate traits in the three species of gazelle (table 2). The horn length FA was negatively related to the percentage of motile sperm and percentage of progressively motile sperm in *G. dorcas* and to the sperm concentration in *G. dama* males. The FA at the greatest oro-aboral diameter at the base of the horn was negatively related to the percentage of motile sperm in *G. dama* and percentage of normal spermatozoa in the three species studied (see figure 2). No significant relationship was found between the FA at the greatest latero-medial diameter at the horn base and any ejaculate trait.

Table 3 presents a summary of the results obtained. The individual levels of inbreeding were found to be inversely related to semen quality only in the species with the highest levels of inbreeding (*G. cuvieri*). The individual levels of inbreeding were reflected by FA both in the species with the highest levels of inbreeding (*G. cuvieri*) and in the species with intermediate levels of inbreeding (*G. dama*). Finally, individual semen quality was inversely related to FA in all the species studied.

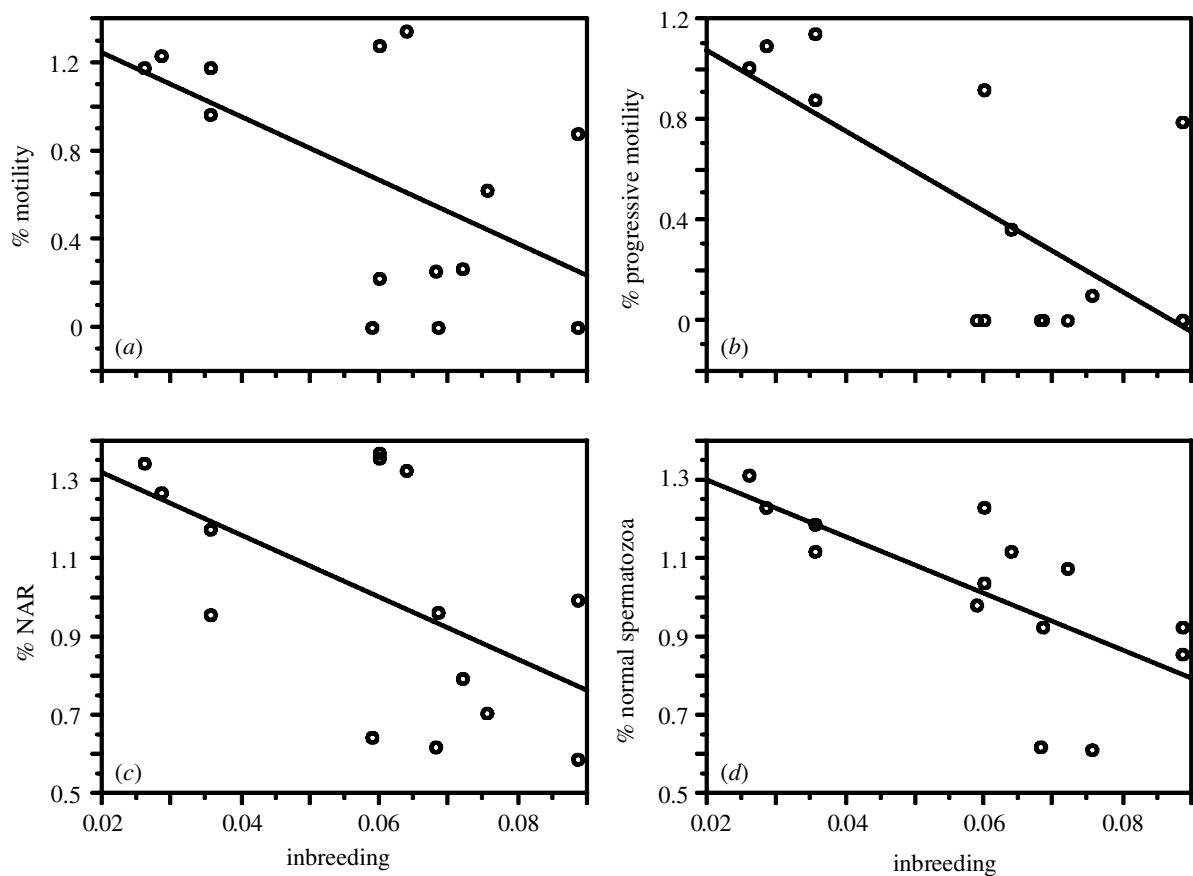


Figure 1. The relationship between inbreeding (log transformed) and four ejaculate traits (transformed into the arcsine of their square root) in males of *G. cuvieri*.

Table 2. Relationship between FA and ejaculate traits in three species of gazelle (genus *Gazella*)

(The results of the Spearman's rank correlations (ρ / p -value) run between the five ejaculate traits and two FA measures in males of *G. dorcas* ($n = 19$), *G. dama* ($n = 16$) and *G. cuvieri* ($n = 13$). FA₁, horn length; FA₂, greatest oro-aboral diameter at the base; n.s., non-significant. See figure 2 for values marked with an asterisk.)

ejaculate traits	<i>G. dorcas</i>		<i>G. dama</i>		<i>G. cuvieri</i>	
	FA ₁	FA ₂	FA ₁	FA ₂	FA ₁	FA ₂
concentration	n.s.	n.s.	−0.64/0.01	n.s.	n.s.	n.s.
% motility	−0.56/0.02	n.s.	n.s.	−0.60/0.02	n.s.	n.s.
% progressive motility	−0.57/0.02	n.s.	n.s.	n.s.	n.s.	n.s.
% NAR	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
% normal spermatozoa	n.s.	−0.50/0.03*	n.s.	−0.65/0.01*	n.s.	−0.56/0.05*

4. DISCUSSION

This is the first study to compare three closely related species with different levels of inbreeding under the same environmental conditions. Our findings indicate that there is a negative relationship between the individual coefficient of inbreeding and semen quality in the species with high levels of inbreeding (*G. cuvieri*) (see also Roldan *et al.* 1998), but not in the species with intermediate (*G. dama*) and low (*G. dorcas*) levels of inbreeding. These results suggest that inbreeding reduces male reproductive function when it reaches relatively high levels. It will be

important to clarify whether this is also the case with the different fitness components which have been found to be influenced by inbreeding, since it could explain why studies carried out on populations with mild levels of inbreeding did not find any significant effects. *G. cuvieri* individuals with high levels of inbreeding had lower proportions of morphologically normal sperm, sperm with intact acrosomes and motile sperm, parameters which are crucial for fertilization success (Soderquist *et al.* 1991; Eggertkruse *et al.* 1996; Donnelly *et al.* 1998). Our findings thus provide support for the idea that inbreeding impairs male reproductive function and

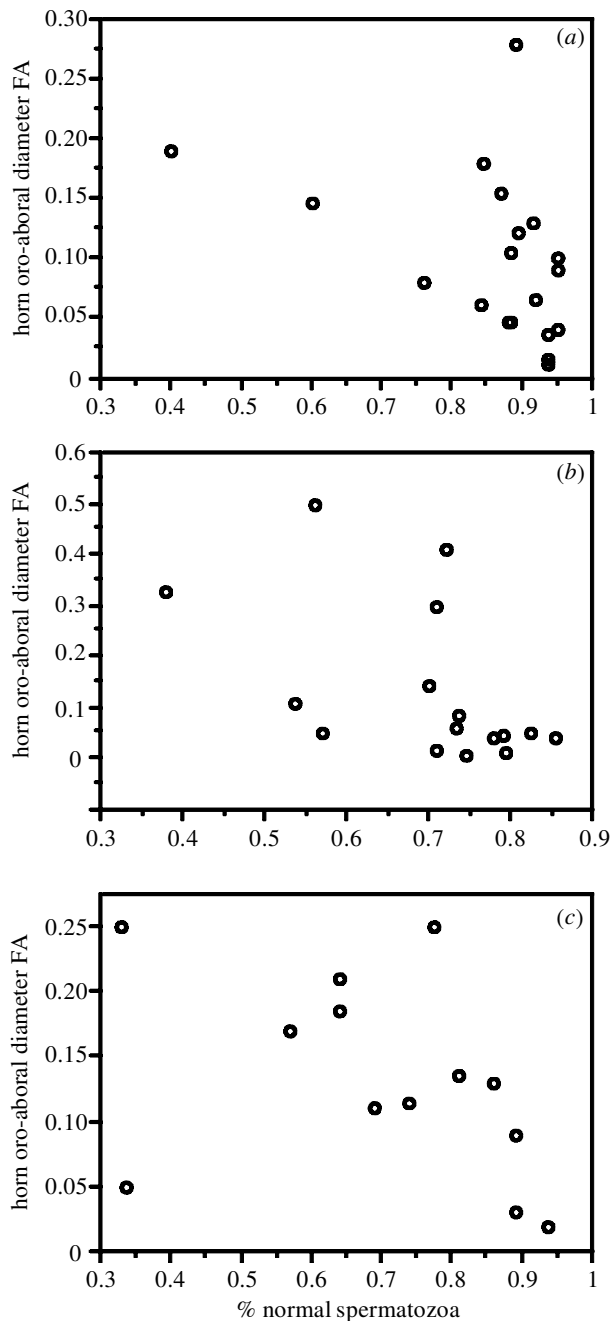


Figure 2. The relationship between FA at the greatest oro-aboral diameter at the horn base and percentage of normal spermatozoa in (a) *G. dorcas*, (b) *G. dama* and (c) *G. cuvieri*.

establish the causal link which interpopulation studies carried out in carnivores have failed to demonstrate (O'Brien *et al.* 1983, 1985, 1987; Wildt *et al.* 1983, 1987; Menotti-Raymond & O'Brien 1993). On the other hand, the finding that inbreeding decreases semen quality in ungulates invalidates the argument that the poor semen quality found in isolated carnivore populations is a common feature of reproduction in male carnivores unrelated to inbreeding (Shields 1993). Given therefore that the effects of inbreeding upon male semen quality seem widespread across taxonomic groups it is worth asking why these particular effects should be so common. Current evidence seems to suggest that inbreeding depression is commonly due to the expression of deleterious recessive alleles or partially recessive alleles that are

Table 3. Relationship between inbreeding, FA and ejaculate traits in three species of gazelle (genus *Gazella*)

	inbreeding/ ejaculate	inbreeding/FA	ejaculate/FA
<i>G. cuvieri</i>	yes	yes	yes
<i>G. dama</i>	no	yes	yes
<i>G. dorcas</i>	no	no	yes

masked in the heterozygote (Charlesworth & Charlesworth 1987; Thornhill 1993; Dudash & Carr 1998). Such recessive alleles are likely to be the result of mutations. Since mutations are much higher in males than in females (Crow 1997) and occur much more frequently in the male germ line (Short 1997), perhaps defects in sperm production (spermatogenesis) are one of the earliest effects evidenced by an increase in inbreeding.

In order to assess the evolutionary implications of the effects of inbreeding upon semen quality, it is necessary to establish to what extent it impairs male reproductive success. In populations in which females are monandrous it would have to be determined whether decreases in semen quality do hinder fertilization success. Perhaps more importantly, in populations in which females are promiscuous, males with reduced semen quality are unlikely to achieve any fertilization success when they face sperm competition (Gomendio *et al.* 1998). Interestingly, sperm morphology and motility (the traits most affected by inbreeding) are the most important determinants of ejaculate success under sperm competition (Gomendio & Roldan 1993; Gomendio *et al.* 1998).

The captive populations examined in our study are kept under very favourable conditions in comparison to the environmental conditions faced by wild populations which may suffer food limitations and exposure to a wide array of pathogens. Earlier studies found that the effects of inbreeding become exacerbated when environmental conditions deteriorate (Keller *et al.* 1994) and a study in which inbred mice were released from captivity into the wild found that the effects of inbreeding were much more pronounced in the wild (Jimenez *et al.* 1994). It is therefore possible that the effects that we have detected in captive populations would become more intense if animals were reintroduced to their former habitat and that, under adverse ecological conditions, mild levels of inbreeding could also have significant effects.

Whether FA is a good indicator of inbreeding or heterozygosity is a controversial issue. The discrepancies could be partly explained by two factors. First, many studies have compared populations and did not control for confounding variables, thus making inferences about causality weak. Second, study populations differ greatly in the levels of inbreeding present. It has been suggested that FA in sexual ornaments could be particularly marked (Møller & Swaddle 1997; Møller & Thornhill 1998), but so far the evidence is contradictory. To the best of our knowledge, the only study carried out in ungulates relating heterozygosity and asymmetry in horns did not find any relationship (Smith *et al.* 1982). Our comparative study shows that FA is positively related to the individual coefficient of inbreeding both in

the species with high levels of inbreeding (*G. cuvieri*) and the species with intermediate levels of inbreeding (*G. dama*), but not in the species with low levels of inbreeding (*G. dorcas*). These findings show that FA in sexual ornaments is a sensitive indicator of inbreeding since it reflects genetic stress before inbreeding is high enough to reduce male reproductive function. Thus, FA could be a valuable tool in detecting mild levels of inbreeding in populations with no known history. This will aid in implementing prevention plans before inbreeding depression actually takes place.

FA seems to be a very sensitive indicator of male reproductive function since it is negatively related to semen quality in the three species studied. Our previous study on *G. cuvieri* has already shown that, at high levels of inbreeding, FA is a good indicator of male reproductive stress (Roldan *et al.* 1998). The findings of the present study showed that this is the case in all the species, irrespective of the levels of inbreeding. Thus, FA in horns seems to be an excellent indicator of male fertility and/or male (sperm) competitive ability, supporting the phenotype-linked fertility insurance hypothesis (Sheldon 1994). The only other supporting evidence for this hypothesis comes from a study in guppies which showed that the male display rate revealed ejaculate characteristics (Matthews *et al.* 1997).

Taken together these findings support the hypothesis that FA in male sexual ornaments is a good indicator of male quality, more specifically of male genetic quality and of male fertility and/or sperm competitive ability. These two traits (male genetic quality and sperm competitive ability) are of fundamental importance to female choice in mammals, since most male mammals provide little more than sperm in terms of investment. Previous studies have shown that female preference is directed towards male traits which provide information about the degree of inbreeding of male bearers (Sheridan & Pomiankowski 1997).

Despite some controversy, a growing number of studies have shown that females do prefer symmetrical males (reviewed in Møller & Swaddle 1997; Møller & Thornhill 1998; Swaddle 1999b). However, many studies have employed experimental manipulations which have resulted in asymmetry values larger than those commonly observed in nature. It also remains to be established whether the magnitude of FA which does occur in natural populations is detectable by females, since the differences between the two sides tend to be small (Swaddle 1999a). A careful consideration of visual perception mechanisms revealed that there are certain features of a trait which can enhance asymmetry discriminatory performance, including the vertical orientation of the plane of symmetry, close proximity of the left and right elements to the mid-line of the body, left and right elements being visible simultaneously, the composition of elements within a trait to form geometrical lines and angles, and the homogeneity of trait contrast and colour (Swaddle 1999b). The design of gazelle horns therefore seems perfectly suited to enhancing symmetry detection by receivers. It seems worth mentioning that FA was more frequent in horn width than in horn length, which could be related to the fact that asymmetries in width may be easier to detect since both sides can be compared simultaneously, while the full

length of the horns is more difficult to be seen simultaneously for both sides from most body orientations.

In conclusion, our study compared three closely related species with different levels of inbreeding and showed that, within each population, inbreeding reduces male reproductive function when it reaches high levels, FA is a reliable indicator of inbreeding that is expressed both at high and intermediate levels of inbreeding, and FA is an even more sensitive indicator of male reproductive stress since the relationship between individual FA and semen quality is present in all the species studied.

We thank Mar Cano for permission to study the gazelles and access to facilities at the EEZA (CSIC), Teresa Abaigar for technical help, electroejaculation of the gazelles and assistance with analyses of the samples, Gerardo Espeso for veterinary expertise and assistance with semen collection, Maria Antonia Barros for assistance with semen collection and analyses of the samples and the staff of the EEZA for expert handling of the animals. We also wish to thank P. A. Parsons and an anonymous referee for their constructive reviews of our manuscript. This study was supported by the Ministerio de Educación y Cultura (DGES Project PB96-0880), Spain.

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